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# PINK1 and MTF2 Modulators Synergize Cell Transplantation Therapy in Parkinson's Disease

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**Abstract.** Parkinson's Disease (PD), the second most prevalent neurodegenerative disease, has the key pathological feature of selective degeneration of dopaminergic neurons (DANs). Current cell therapy based remedy of PD centered on DAN transplantation, whether stem cell derived, or from fetal tissues, yet the possible dysfunction underlying in the microenvironment and non-neuronal mechanisms may impede this solution. The unresolved question is if microenvironment impairments and other non-cell-autonomous signaling may affect the healthy, transplanted DANs, triggering survival rate reduction. We hypothesized that non-neuronal mechanisms do exist thus specific drugs should be incorporated along with DANs during transplantation. Here we performed single cell RNA sequencing (scRNA-seq) analysis using Seurat and AI based package Monocle3 to confirm the existence of non-cell-autonomous signaling and revealed PINK1 and MTF2 as the main culprits through the comparison of initial differentiation dataset and long term transplantation dataset of transplanted DANs. Targeting PINK1, we identified the drug MTK458 and validated its effectiveness through AI based drug screening and molecular docking. Together, these findings supported MTK458 to be an effective co-transplant material designated for alleviating the abnormality in non-neuronal mechanisms or microenvironment.

**Keywords:** Parkinson's Disease, Co-Transplantation, Cell Therapy, Dopaminergic Neuron, Bioinformatics, Single-cell RNA-Sequencing, K-Nearest Neighbors, AlphaFold 3, Machine Learning.

## 1. Introduction

Parkinson's disease (PD) asserts its prominence as the second most pervasive neurodegenerative disease, affecting more than 8.5 million people worldwide by 2019 [1]. Patients often manifest motor symptoms — tremor, rigidity, slow movements and balancing difficulties — as well as non-motor symptoms like sleep disruption (Figure. 1A). PD patient's neurological analysis found an accumulation of Lewy bodies formed by α-synuclein deposition and loss of dopaminergic neurons (DANs) in midbrain substantia nigra pars compacta, and ventral tegmental area [2] (Figure. 1B). The loss of DANs is the direct contributor to PD's symptoms, and thus is the key for devising a viable treatment.

For decades, standard treatments focused on dopamine replacement therapy (DRT) to regulate levels of dopamine, most commonly using the dopamine precursor L-DOPA penetrating the blood-brain barrier to elevate dopamine levels [3]. Emerging therapies include deep brain stimulation therapy (DBS), employing electrical impulses to restore abnormal neural activities [4]. However, neither of these PD treatments tackles the root factor of dopaminergic neuron loss. Despite PD's proliferating cases, these

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current clinical treatments are said to be palliative: remedial actions that do not inhibit the process of degeneration. In the search for more fundamental treatments, cell therapies, involving transplantation of cellular materials into patients, uncovered a new approach of restoring degenerate neurons.

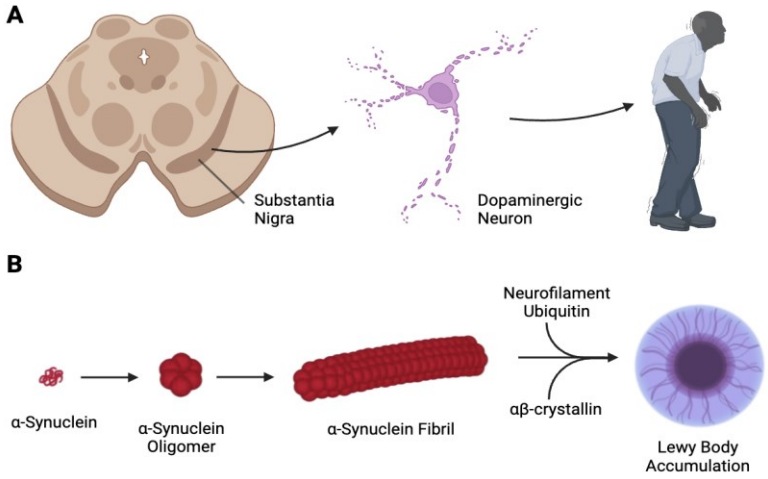


**Figure 1.** Biological Mechanisms of PD. (A) Loss of dopaminergic neurons. (B) α-synuclein deposition and formation of Lewy Bodies. Modified based on Goldoni et al., 2022.

While DAN transplantation gained a surge in popularity in recent years, the unresolved difficulty of diminished cell survival rate, typically lower than 10% in both autologous and allogeneic models, can potentially render it ineffective [5]. The low survival rate of DAN transplantation is the major hurdle, commonly due to the selective vulnerability of transplanted DANs and contaminating cell types. However, a cause typically overlooked is microenvironment or non-cell-autonomous signaling: the disease-causing agent might not be only within dopaminergic neurons, but also be lurking in nearby cells and extracellular matrix.

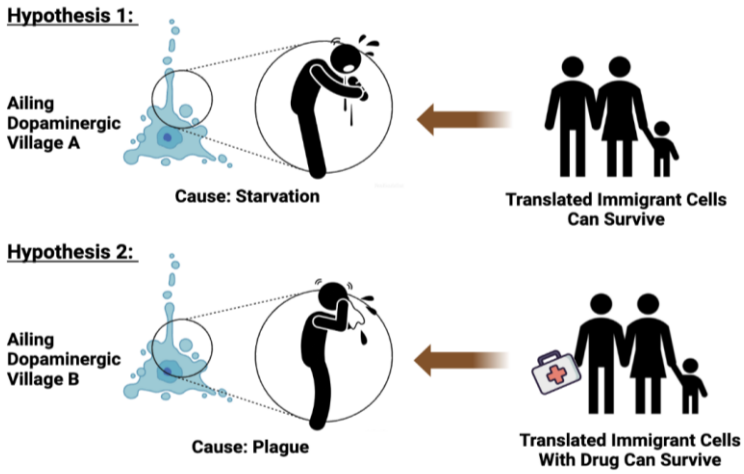


**Figure 2.** Two Hypotheses of PD emergence and cure. To present two hypotheses of what causes the low DAN survival, an analogy of the disease environment as an ailing village is displayed. First, the cause might be "famine", then new immigrants will not be affected by indigenous villagers. Second, if the issue is "plague", the immigrants will have to bring medicines. If PD derived from purely DAN dysfunction, implanting more DANs can be a remedy; if not, adjustments within the microenvironment using drugs have to be made.

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Our main outstanding question is: what limits the survival rate of DANs? We hypothesized that there is a defect in the microenvironment, rendering the strategy of only replenishing DANs insufficient (Figure 2). To address this hypothesis, there are two questions to be answered: first, whether a non-neuronal mechanism exists, affecting the transplanted DANs, and second, what molecules can compensate for such effects and boost the survival of DAN transplant. Thus, a co-transplanted drug should be added to alleviate the defects in the microenvironment. Here, we utilized multiple scRNA-seq datasets of PD and with best suited machine learning based tools, we revealed critical genes through comparison between initial differentiation and long-term transplantation data, thus proposing drugs as a co-transplanted material to address the challenge in cell transplantation by targeting the microenvironment dysfunction.

## 2. Methods

### *2.1. Dataset Overview*

In order to address our hypothesis, we employed the comparison between disease versus healthy datasets to confirm the existence of non-neuronal signaling in the PD microenvironment. We then utilized data comparison between differentiation versus transplantation datasets to observe the differences before and after transplantation, and further compared it with the natural development dataset to reveal specific genes that could be causing this dysfunction. We also integrated an additional midbrain organoid scRNA-seq datasets for further comparative analysis.

*2.1.1. Disease versus Healthy Datasets.* We employed the open source single cell RNA sequencing (scRNA-seq) dataset from Khan et al., 2021 (GSE187012), which utilized a combined method of neurotoxin maneb and paraquat to model PD mice. A healthy control group (GSM5667021), without the injection of neurotoxin, and an PD disease modeled experiment group (GSM5667021) were both investigated in this study. Both groups record the gene expression levels in substantia nigra pars compacta at single cell resolution. We employed these two datasets to analyze the gene expression differences in non-neuronal cells and for pathway examination.

*2.1.2. Differentiation versus Transplantation Datasets.* Datasets from Tiklová et al. (2020)[6] were used to compare gene expression between the course of DAN differentiation and transplantation. PD rats, modeled with neurotoxin 6-hydroxydopamine (6-OHDA), were transplanted with fetal tissues and human embryonic stem cells (hESCs) derived neuronal grafts in ventral midbrain. The differentiation dataset (GSE132758), recording the differentiation stage of early transplantation, and the transplanted dataset (GSE118412), measuring the expression in transplanted graft after long term survival, gave insights into how transplanted DANs were affected by non-cell-autonomous mechanisms.

*2.1.3. Analysis of Developing Brain Gene Expression By Braindex.* We implemented a developmental database to compare and consolidate our results. Braindex, a brain development expression portal created by Kleinman Lab, profiles gene expression of mouse brain in conditions of cancer and neuro-development at single cell resolution [7]. We employed this database to obtain the gene expression across developmental stages to examine which of our target genes revealed by scRNA were more significant, by comparing its trend during healthy development, and during the transplantation process of growth.

### *2.2. Single-cell RNA Sequencing Analysis*

*2.2.1. Pre-processing and Cell Type Identification.* Our analysis is initiated via the R package Seurat V5, tailored specifically for single-cell RNA-sequencing (scRNA-seq) data investigation. To filter out low-quality cells and increase the accuracy of downstream analysis, we began by performing quality control, removing cells with unique feature counts fewer than 500, and mitochondrial counts more than

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5%. The data from different rats in the same database are then integrated, and normalized using "LogNormalize" to stabilize variance. After normalization, 2000 highly variable features were identified using "FindVariableFeatures". These variable features were scaled using "ScaleData" to prevent the highly expressed genes from overshadowing others. Next, dimension reduction was applied using Principal Component Analysis (PCA), capturing the source of variability in the dataset. Finally, we clustered the cells with "FindNeighbors" and "FindClusters", categorizing cells by gene expression and displaying visually using Uniform Manifold Approximation and Projection (UMAP). These cell clusters were further analyzed using "FindMarkers" to reveal marker genes for the purpose of identifying their specific cell types. These cell type identifications were reinforced with violin plots of corresponding marker genes. This same procedure was implemented on all five datasets before their individual analysis.

*2.2.2. Trajectory and Pseudotime Analysis.* The machine learning package Monocle3 was used on top of Seurat to perform trajectory and pseudotime analysis for a more comprehensive comparison between differentiation and transplantation data. We transferred the data accessed by Seurat to a cds object in Monocle3, so that pre-processing and cell type identification don't need to be reiterated. The same clusters were visualized in Monocle3 for double check, then the trajectory was learnt by using the machine learning function "learn_graph", displayed on the same UMAP. Furthermore, the cell types were ordered in pseudotime by "order_cells", visualized in color from yellow to purple, indicating the relative time of differentiation progression. Ultimately, box plots of pseudotime of cell types were plotted with ggplot2, an R visualization package.

*2.2.3. Gene Set and Pathway Analysis.* Via the R package clusterProfiler, we performed gene set enrichment analysis to interpret the importance and functions of specific pathways. We created a gene list using markers from the seurat object along with the marker gene expression and Log2 Fold Change. Then we employed functions "enrichGO" and "enrichKEGG" to carry out functional enrichment analysis. Furthermore, we executed gene set enrichment analysis using "gseGO", obtaining ridgeplots and dotplots to analyze the importance of each pathway.

### *2.3. Drug Mining Tools*

We target significant genes revealed by scRNA-seq and the developmental database with the most advanced tool currently available, the machine learning based tool AlphaFold 3 for protein structure visualization and SwissDock for molecular docking. We also compared the protein structure in the Protein Data Bank (PDB) and predicted by AlphaFold 3 during drug docking.

*2.3.1. AlphaFold 3.* As a deep learning based model for predicting protein structure, AlphaFold3 performs in high accuracy with nearly every protein in Protein Data Bank (PDB). AlphaFold 3 utilizes pair-wise representation of chemical complexes and generates their atomic arrangements, allowing it to perform highly in predicting protein structures and interactions.

*2.3.2. SwissDock.* In addition, molecular docking was done to explore how effectively the drug targets the gene. To achieve the goal, I utilized SwissDock, a docking program using EADock ESS engine to analyze how a ligand and a protein forms a stable compound [8]. Generating and ranking the binding models with solvent effects and CHARMM energies, the algorithm enables SwissDock to perform accurate docking within minutes.

## 3. Results

### *3.1. Verifying Non-neuronal Mechanisms in PD*

According to our hypothesis, the first question is whether only refilling DANs is sufficient to relieve the defects in brain regions affected by PD. Are the defects only purely DAN-derived or are they also caused by other non-neuronal glial cells and extracellular matrix? To approach this question, we explored the

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non-cell autonomous pathogenic mechanisms of PD. In order to verify, we found datasets of PD disease-modeled condition and healthy conditions. By comparing the gene expression in microglia, oligodendrocytes, and astrocytes instead of dopaminergic neurons via scRNA-seq, we revealed a large disparity in the microenvironments in disease versus healthy state, thus suggesting that non-cell-autonomous signaling certainly contributed to the disease. The PD disease dataset (GSE187012) [9], which contained a healthy control group, and a disease modeled experimental group treated with maneb and paraquat, was analyzed to uncover nine different cell types each, visualized by UMAP reduction, each cluster representing a unique cell type or subtype (Figure 3). These cell types were identified using their associated genes. Notably, not only two cells related to dopamine production, dopaminergic neurons (DANs) and dopaminergic progenitor cells (DPs), were unveiled, but also, two subtypes of microglias were discovered in both groups: HM (homeostatic microglia) and DAM (disease-associated microglia). Specifically, DAM, a type of microglia related to neurodegenerative diseases may suggest abnormality within the microenvironment. Moreover, neuroblastoma cells, a type of malignant cancer cell, were also detected.

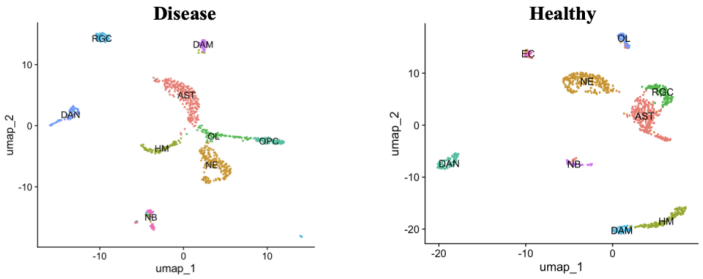


**Figure 3.** Cluster Diagram with UMAP reduction in Healthy and Disease Data.

Remarkably, we found disease related differences between the microenvironments of disease and healthy conditions by analyzing the expression of PD associated genes in non-neuronal cells (Figure 4). FBXO7, which is typically in deficiency in PD models causing impaired mitochondrial function, is also expressed in low levels in oligodendrocytes of disease-modeled dataset, but high in healthy dataset. SYNJ1, found to be reduced in MPTP-induced Parkinson mice, is also expressed in low levels in DAMs of disease-modeled dataset compared to the healthy dataset. SYT11, on the other hand, reported to be accumulated in PD mice, is found in high levels in astrocytes of disease-modeled dataset while low in healthy dataset.

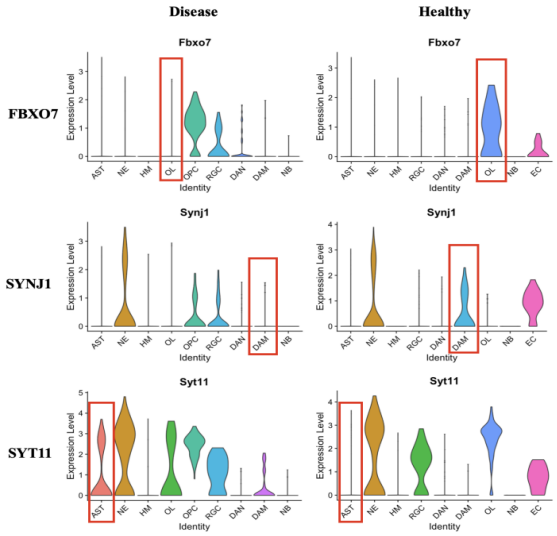


**Figure 4.** Comparing Disease Associated Genes in Microenvironment

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In the end, the differences in expression levels of disease associated genes in non-neuronal cells suggests differences occurring within the microenvironment. This could contribute to microenvironment dysfunction that leads to degenerative death of healthy dopaminergic neurons. Therefore, we arrive at the finding that it may not be sufficient to just restock the DAN for PD treatment. This microenvironment or non-cell autonomous role in PD has been reported previously on glial cells, supporting our results. Microglia, especially DAMs, for example, have been found to play damaging roles in neurodegenerative diseases, and are especially pivotal in exacerbating neuroinflammation and autophagy impairment [10].

### *3.2. Comparing Differentiation and Transplantation Data of PD*

As demonstrated in 3.1, PD is indeed associated with non-neuronal cells and microenvironment abnormality. This raised the second question: would this malfunctioning microenvironment cause the healthy DANs transplanted to be affected? To further investigate, we compared the differentiation and transplantation conditions in a 6-hydroxydopamine PD model transplanted with ventral midbrain grafts to examine the change in gene expression in DANs during and after transplantation. We utilized two datasets, one differentiation dataset (GSE118412) and one transplantation dataset (GSE132758) [6], which were generated from the same lab to reduce variance. Using scRNA-seq, the specific cell types of each dataset are revealed before identification of specific disease-causing pathways.

*3.2.1. Identifying Cell Types in Differentiation and Transplantation Data.* In the differentiation dataset, seven distinct cell types were disclosed using their corresponding cell marker genes, plotted in a UMAP cluster plot (Figure 5). Similarly, eight cell types in the transplantation data were revealed. Noticeably, two types of cell related to dopamine production, dopaminergic neurons and their progenitor cells, were uncovered using the two marker genes: TH, crucial to synthesis of dopamine, and NR4A2 (also known as Nurr1), essential for development and maintenance of dopaminergic neurons. After further analysis with mature neuronal markers and markers of progenitor cells, DANs and DPs could be distinguished.

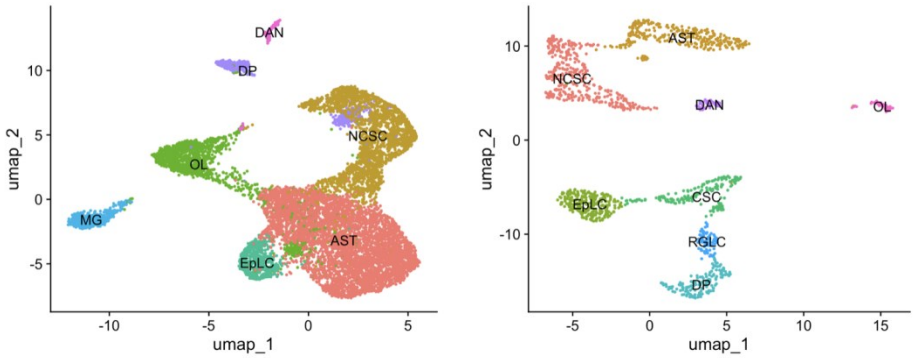


**Figure 5.** Differentiation VS Transplantation Cell Clusters with UMAP visualization. Left: Differentiation; Right: Transplantation. The identified cell types are exhibited using the dimension reduction technique of Uniform Manifold Approximation and Projection (UMAP) in a cluster diagram, with each cluster and color representing a unique cell type

*3.2.2. Trajectory Analysis of Differentiation and Transplanted DANs.* To further compare differences within differentiation and transplantation dataset, we conducted trajectory analysis, visualized by UMAP (Figure 6), and plotted their relative pseudotime with a box plot (Figure 7). We found a normal progression in the differentiation dataset, with DAN emerging after DP at the latest stage. Surprisingly, we discovered abnormal progression in transplantation data: there are other cell types between DAN and DP. This might indicate defects within DANs after long-term transplantation, potentially caused by microenvironment dysfunction or non-cell-autonomous mechanisms.

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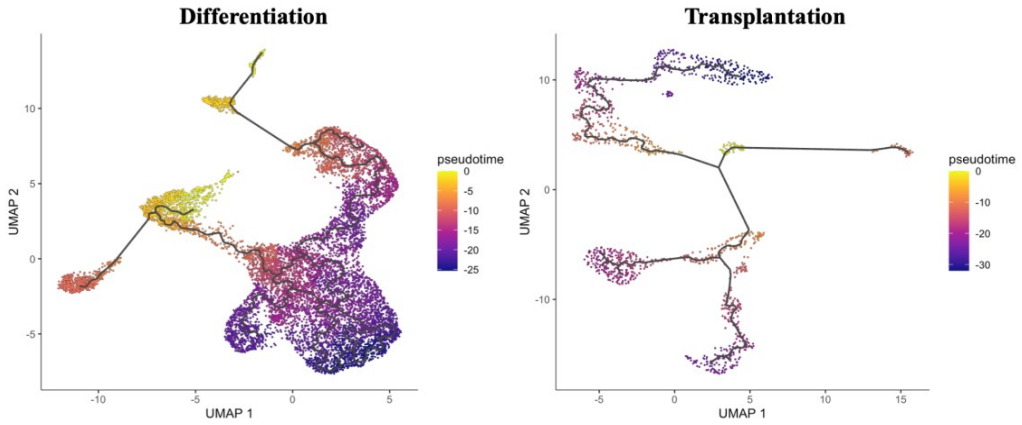


**Figure 6.** Trajectory Analysis of Cell Types. Left: Differentiation; Right: Transplantation. The color indicates the progression of cell types, with purple the earliest, yellow the latest.

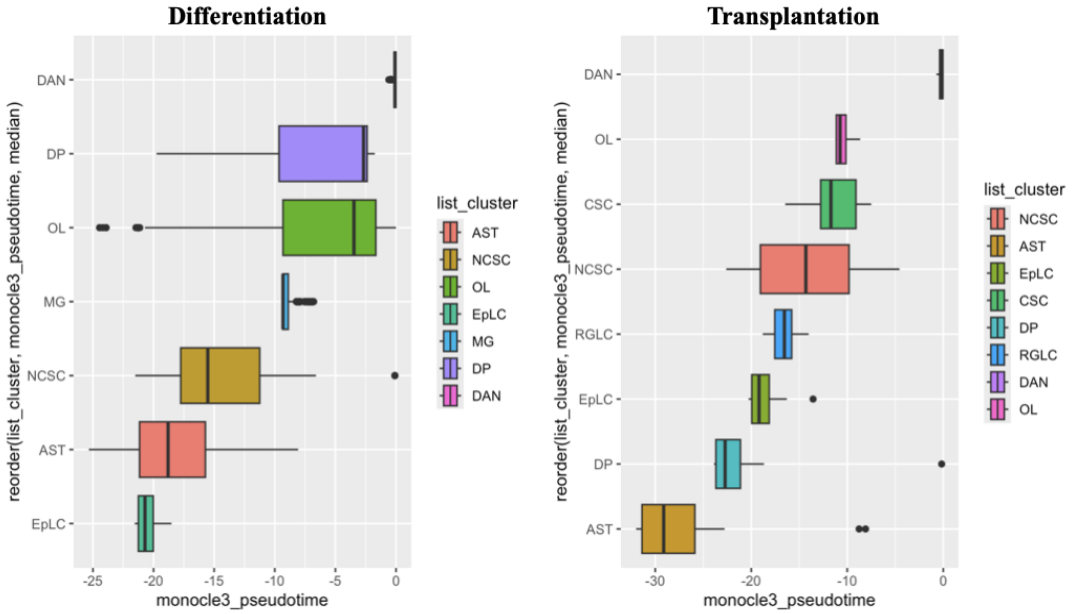


**Figure 7.** Box Plot of Pseudotime Data. Left: Differentiation; Right: Transplantation.

*3.2.3. Gene Set Enrichment Analysis.* In order to further confirm our hypothesis that the abnormality lurks in the transplantation microenvironment, we compared pathways by using gene set enrichment analysis of the differentiation and transplantation data. Using a ridgeplot showing the distribution of enrichment across gene sets, we surmise that dysfunctions or abnormalities may underlie within the transplantation microenvironment (Figure 8). While the differentiation dataset contains gene sets with variable enrichment distribution, the gene sets in the transplantation dataset show similar enrichment distribution. This difference may be attributed to microenvironment dysfunction or other defects in non-neuronal mechanisms. Moreover, surprisingly, by using a dotplot of activated and suppressed gene sets, we discovered that although both datasets show traits of activated differentiation pathways, the transplantation dataset shows a higher presence in mitotic transition, metaphase and anaphase, and nuclear division that parallels cell proliferation (Figure 9). This might indicate gene dysregulation and other issues, such as cancers.

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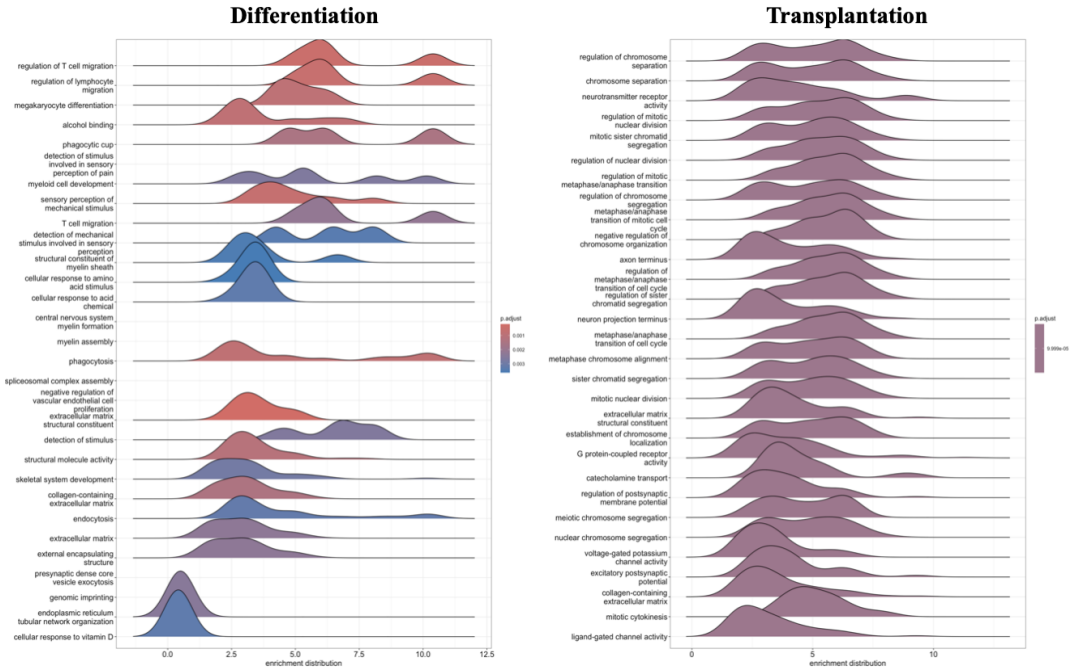


**Figure 8.** Ridgeplot of Gene Set Enrichment.

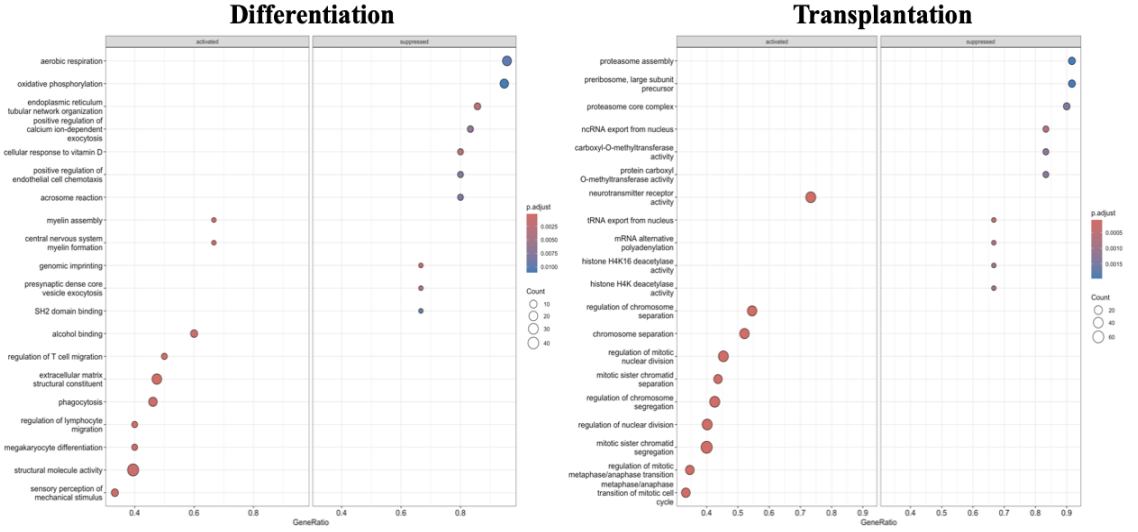


**Figure 9.** Dotplot of Activated and Suppressed Gene Sets.

*3.2.4. Gene Expression Differences in Differentiation and Transplantation.* We compared the expression of PD related genes in differentiation and transplantation dataset to reveal differences that could be caused by non-neuronal mechanisms. Fifteen genes (PINK1, PRKN, GBA, PARK7, MTF2, PPP6R2, ATP13A2, VPS35, MAPT, SYT11, SNCA, ADD1, IRS2, USP8 and USP25) were chosen from GWAS [11]. By comparing these disease related genes in the two datasets, inconsistency was found with genes PINK1, PARK7, PRKN, GBA, MTF2 and PPP6R2 (Figure. 10), while the other nine genes exhibit little differences. While PINK1 and PARK7 are highly expressed in differentiation and lowly in transplantation, the other four genes display the inverse.

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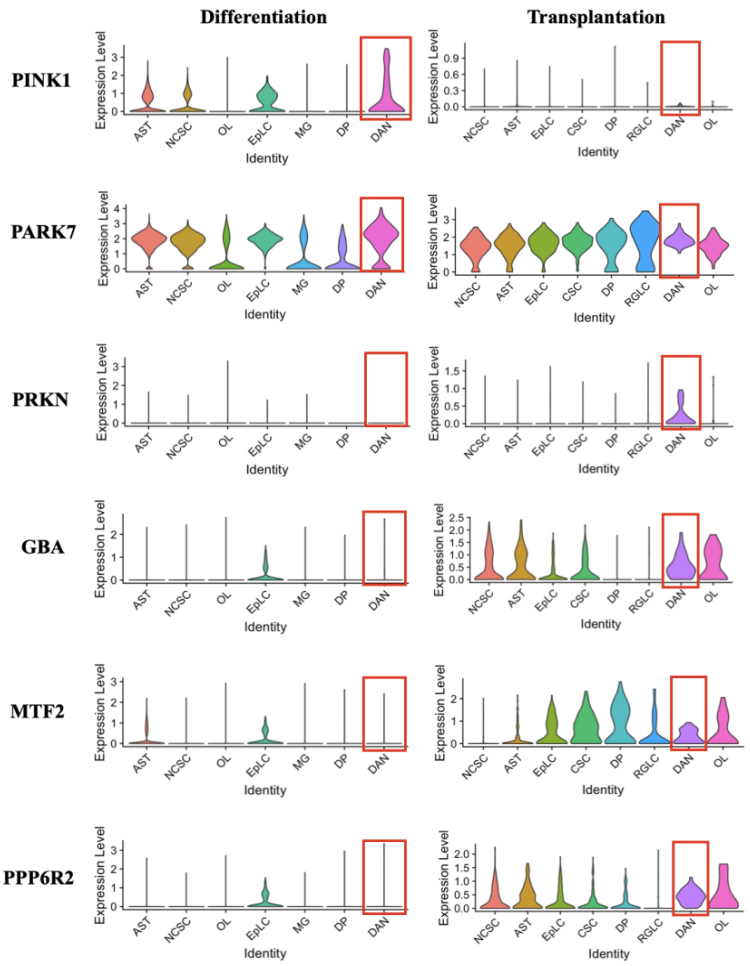


**Figure 10.** Expression levels of Six Significant Genes in Differentiation and Transplantation

Within the six genes found to show differences, deficiency of PRKN and GBA were typically found in PD, as PRKN deficiency harms mitophagy pathways and GBA insufficiency could damage the autophagy-lysosome system [12,13]. PINK1 and PARK7 on the other hand are more complicated: their mutations were not directly associated with higher or lower expression levels. Mutations in PINK1 are associated with disrupted mitophagy, while mutations in PARK7 could trigger the gene to be oxidized, which is a biomarker of PD, and cause oxidative stress [14]. Dysregulation in PINK1, Parkin and PARK7 are all associated with Early Onset Parkinson's Disease (EOPD) [15]. MTF2 and PPP6R2 are novel genes, and it is not known whether it was upregulated or downregulated in PD, nor is it known about their specific roles in the disease. The drastic differences shown in these six genes between differentiation and transplantation data indeed confirms that transplanted cells were affected, or even potentially damaged by the impairments in non-neuronal signaling.

### *3.3. Characterizing Critical Genes and Pathway*

*3.3.1. Filtering the Key Genes underlying abnormality in Transplantation.* As shown in 3.2, there are critical differences between transplantation and differentiation, and there are abnormalities in the transplantation data. This result suggests that the healthy DANs transplanted may be influenced by the dysfunctional signals in the microenvironment. But what are the most crucial genes causing this influence? Among the six genes found, we compared the trend of expression in the differentiation and transplantation data to the trend of development in the natural growth phase. In the end, the trend in

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differentiation and transplanted versus general healthy developmental trend in the four genes, PRKN, GBA, PARK7 and PPP6R2, did not display significant differences, suggesting that the gene expressions were not severely affected by microenvironment and non-neural dysfunction. However, differences in general trend was observed in PINK1 and MTF2. The expression levels of PINK1 decreases from differentiation to transplantation, which is in contrast with the developmental trend in the healthy environment that shows increase in expression along with growth. Similarly, the expression of MTF2 increases in differentiation to transplantation but decreases in general healthy growth trend (Figure 11).

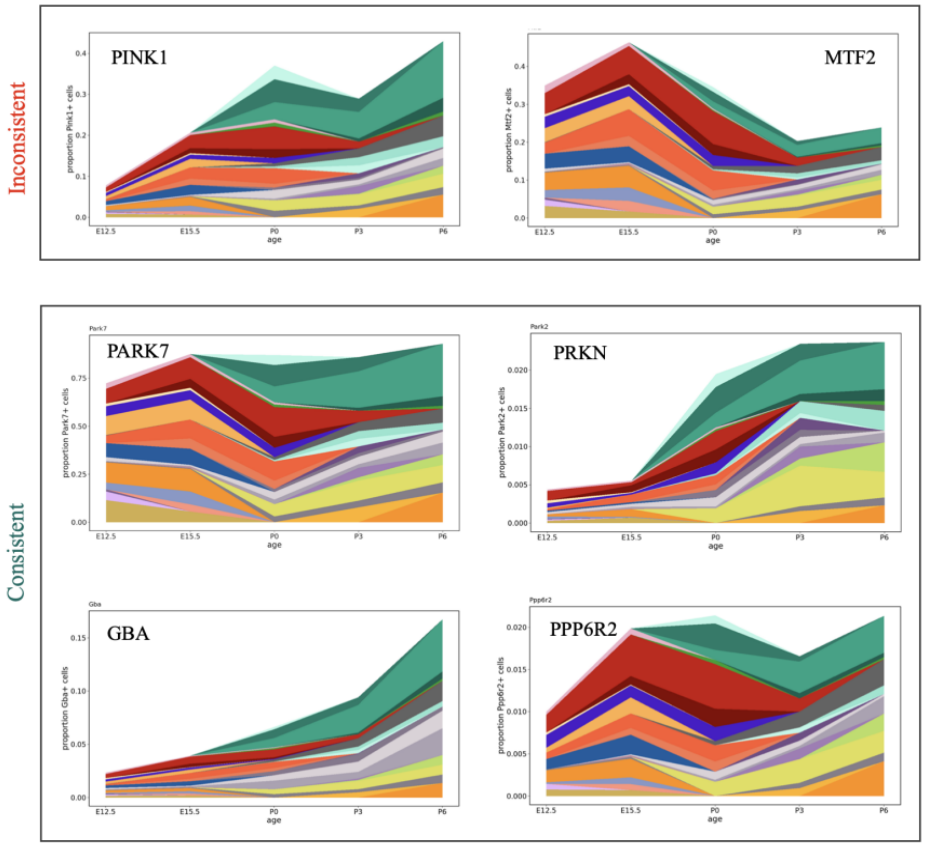


**Figure 11.** Gene Expression Trend of PINK1, MTF2, PARK7, PRKN, GBA and PPP6R2 in Brain Development visualized by Braindex [7]. The x-axis shows age, the y-axis shows the proportion of gene positive cells, and colors indicate different cell types. The inconsistency of PINK1 and MTF2 between differentiation to transplantation trend and general development trend reveals that defects might be lurking within their pathways, and that they might be the main culprits in reducing the survival of transplanted DANs. Thus, they are chosen for further analysis.

*3.3.2. Gene Regulatory Pathway Analysis.* As detailed in 3.3.1, PINK1 shows a decreasing trend during transplantation and increasing trend in healthy development, whereas MTF2 has an inverse pattern. Therefore, we hypothesized that there is a negative regulatory relationship between them which might be caused by a linked pathway. The first question when tackling this hypothesis is whether a negative correlationship exists. Through the use of a pseudotime plot, we discovered a strong opposite trend of PINK1 and MTF2 expression in both healthy and disease microenvironments (Figure 12). However, fluctuations in expression in disease microenvironment are drastically larger than they are in healthy microenvironment, which may indicate that their pathways play important roles in PD.

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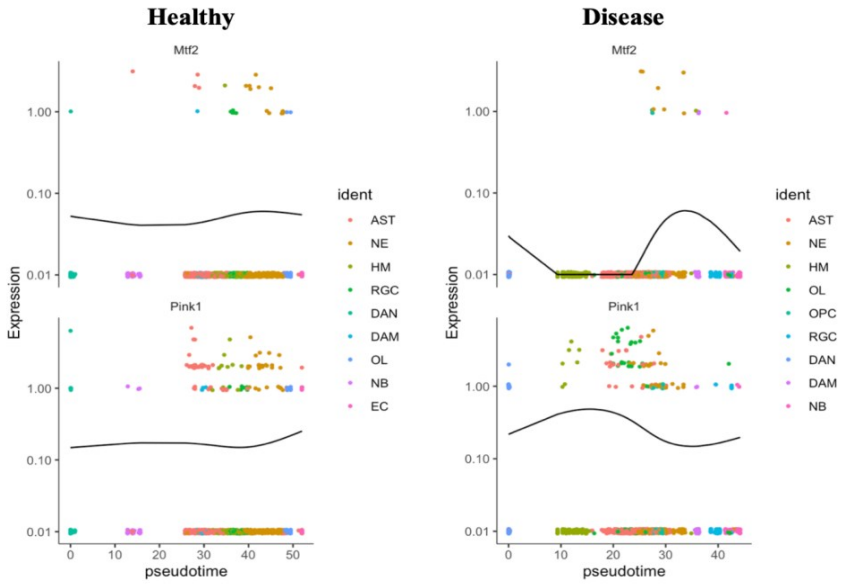


**Figure 12.** Pseudotime Expression of MTF2 and PINK1.

To uncover their linked regulatory pathways, we utilized the String network to reveal associated genes (Figure 13). We discovered the gene EED, which has a direct link to PINK1 while also interacting with a myriad of genes linked to MTF2. EED is a key component of PRC2, and plays a prominent role in growth, self-renewal and differentiation of stem cells; in the mammalian neural system, it could regulate neuronal differentiation and neurogenesis [16]. Though the effects of EED on PD related genes is unclear, it can give insights into a novel pathway of neurodegeneration.

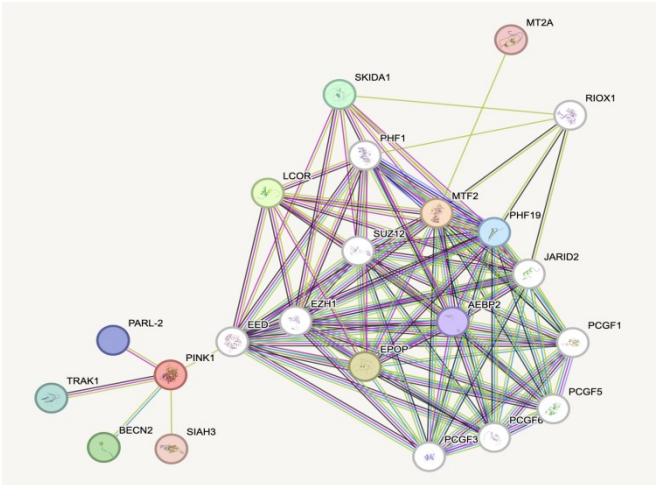


**Figure 13.** Relationship between PINK1 and MTF2 with String network.

To further unearth what specific issues causes the dysregulation in PINK1, genes reported upstream to PINK1 are analyzed. In healthy mitochondria, PINK1 are cleaved in the inner mitochondrial membrane (IMM), a process facilitated by PARL, leading to PINK1 degradation. UBR1, a gene binding to N terminals of PINK1, assists degradation. The comparison between differentiation and transplantation dataset suggests that PARL and UBR1 both have notable changes during the course of transplantation in DANs and other non-neuronal cells in the microenvironment like oligodendrocytes, which may suggest potential disturbances (Figure 14).

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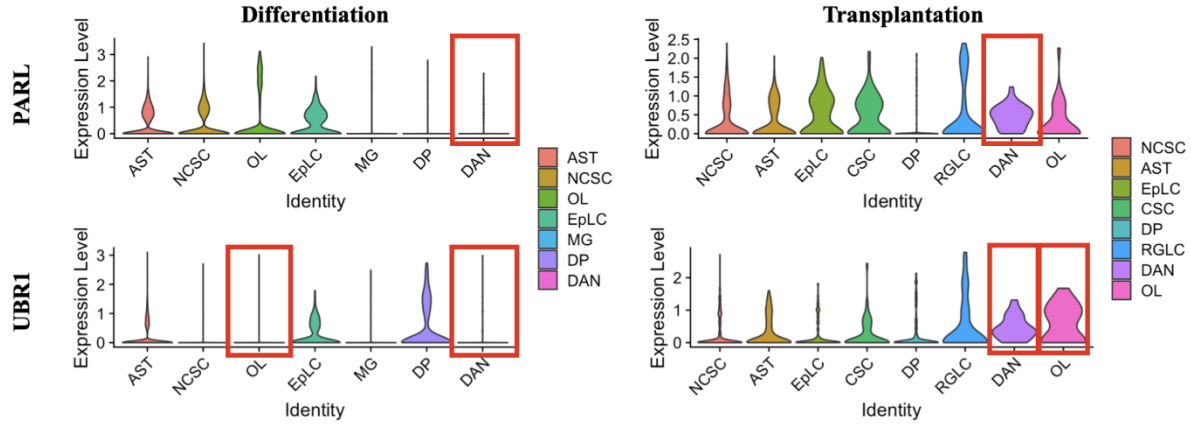


**Figure 14.** Expression Level of PARL and UBR1 in Differentiation and Transplantation.

### *3.4. Drug Mining*

As suggested in 3.3.2, PINK1 and MTF2 are negatively correlated. Between them, ultimately, PINK1 was chosen for drug screening (Figure 15), because MTF2 is a novel gene coding a transcription factor: there is a lack of information to its novelty and moreover, transcription was often considered "undruggable" due to its disordered essence.

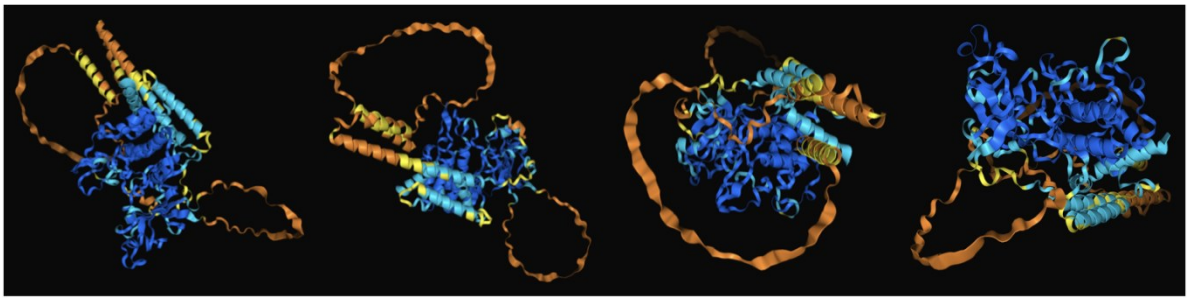

**Figure 15.** Protein Structure of PINK1 Revealed by AlphaFold3. The colors dark blue, light blue, yellow and orange indicate a decrease in confidence level respectively.

Previous results point to a drug activating PINK1, as PINK1 levels decreased during transplantation, inconsistent with the increasing expression in natural healthy development. We found three confirmed molecules that successfully activate PINK1: N6-Furfuryladenine, a neo-substrate kinetin [17]; MTK458, a small molecule mitigating pUb and α-synuclein build-up [13]; and Niclosamide, an anthelmintic drug that temporarily disrupts the mitochondrial membrane potential [18].

Using Swissdock, how firmly the connection formed by PINK1 and the drugs was revealed. While N6-Furfuryladenine has the maximum affinity of -5.845 kcal/mol (Figure 16A) and Niclosamide with -5.575 kcal/mol (Figure 16A), MTK458 with -8.256 kcal/mol (Figure 16A) is the drug docking the closest. Dimethyl Sulfoxide, a versatile solvent used as a negative control, has an affinity of -1.900 kcal/mol (Figure 16D).

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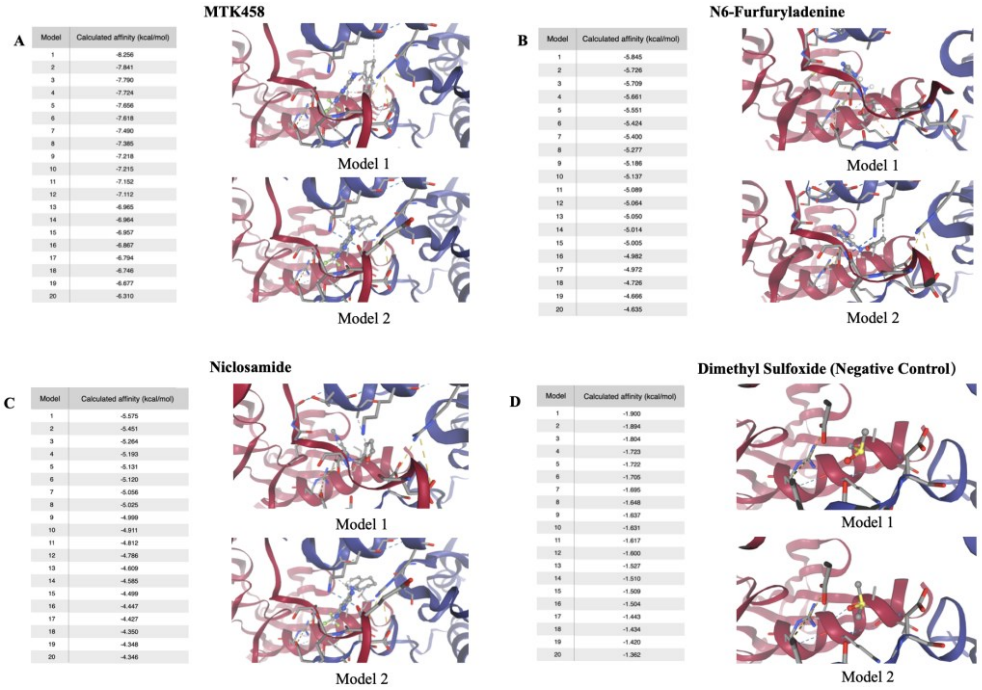


**Figure 16.** Molecular Docking of PINK1 from PDB with MTK458, N6-Furfuryladenine and Niclosamide. Each panel shows the twenty locations of docking with the greatest affinity for each drug, with a smaller calculated affinity number in kcal/mol signifying greater affinity. The dash line indicates interactions, hydrogen bonds in blue, ionic interactions in yellow, cation pi interactions in orange, and hydrophobic contacts in gray.

## 4. Discussion

### *4.1. Limitations*

This study is novel and important by shedding light on a new co-transplantation strategy to enhance the survival of dopaminergic neurons, but there are a few minor reservations. Firstly, for the purpose of identifying associated genes, we compared differentiation versus transplantation dataset. However, to reduce variance, we chose two datasets from the same lab, which could result in a caveat. If biases occur within this lab, it could result in systematic errors. Despite these potential flaws, we were unable to find a more faultless and comprehensive system. Thus, we utilized a natural developmental database to compare as well to reduce the potential sequencing biases. In the future, as more differentiation and transplantation data are gathered, these datasets can be integrated for multimodal analysis, increasing reliability and revealing more drug targets for co-transplantation. Secondly, transplantation datasets used in this study were obtained by mouse or rat models, but not on PD patients, since postmortem patient samples for scRNA-seq are not available. If pathways are different in rats, mice and humans, then the identified drugs might not effectively target the identified genes. Dealing with limited human datasets, spatial transcriptomics using slide-seq can bridge the gap when translating from animals to humans.

### *4.2. Laboratory Validation*

To test our conclusions, we propose a co-transplantation experiment, combining MTK458 with human iPSC derived DANs to be transplanted in PD modeled mice. Long term survival rates of DANs with drug co-transplantation could be compared with conditions with sole transplantation, while further scRNA-seq analysis could be used to test the expression of PINK1 and associated genes in DANs and cells within the microenvironment. However, using immortalized cell lines in this approach might not be suitable, because they can not fully capture the features of DANs.

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Another method to be employed is using the gene editing tool CRISPR (Clustered Regularly Interspaced Short Palindrome Repeats) to filter the six genes identified in 3.2.3, and reveal which are responsible for low survival rates of DANs during transplantation. Using CRISPR, the six genes could be deactivated sequentially in cultured DANs, generating different cell lines, each with a distinct gene knockout. Subsequently, by transplanting the edited DANs into mouse or rat models, if DANs with specific gene disabled exhibit higher or lower survival rates than others, these genes can be indicated as important. These significant genes could be further analyzed by targeting their pathways and associated proteins using drugs, enhancing their activation or increasing their degradation, to observe if survival rates are improved. Side effects of drugs such as inflammation could also be experimented, by necropsy and tissue analysis [5].

### *4.3. In Vivo Reprogramming as Alternative Solution*

Targeting non-neuronal mechanisms with PINK1 related pathways could potentially enhance the survival rate of dopaminergic neurons not only in transplantation therapy, but also during in vivo reprogramming. In vivo reprogramming is critical for rendering differentiated cells as dopaminergic neurons in the patients' midbrain, alleviating symptoms by remedying the degenerative death of DANs. Astrocytes had been suggested to have the ability to be reprogrammed to DANs via a one-step conversion of diminishing RNA-binding protein PTB [19,20], though disputes regarding the identity of reprogrammed cell emerged [21]. This solution bypasses the need for transplantation, however, once new DANs were produced by reprogramming, their survival may also depend on the health of its microenvironment. By using drugs revealed in here, non-neuronal defects may be improved, thus enhancing the efficacy and rate of success of in vivo reprogramming.

### *4.4. Future implications*

This study demonstrates a range of implications for DAN transplantation therapy. First, PINK1 reduction can be caused by different genetic mutations or environmental factors. PINK1 has been reported to be regulated by multiple pathways and molecules, including insulin and small interfering RNAs (siRNA) [22,23]. Further identification of biomarkers through genetic profiling of individual patients in personalized treatments could pinpoint the root agents causing dysregulation of PINK1 to address their personal needs. This can be coupled with autologous iPSC derived DAN transplantation to maximize personalization. PD is highly heterogeneous, driven by issues within multiple systems, neurotransmitters and genes, thus genetic variability between individuals could affect treatments significantly.

Secondly, PINK1 dysregulation found in this study could suggest how mitochondrial dysfunction may regulate the survival of transplanted dopaminergic neurons. Defects in PINK1-parkin pathway is the most common cause of Early Onset Parkinson's Disease (EOPD), due to the disrupted mitophagy leading to neuronal damages [15]. Prolonged mitochondria loss could cause insufficient ATP production, cellular toxicity, accumulation of reactive oxygen species (ROS) and calcium current abnormality, triggering neuronal loss [24].

Thirdly, organoids could also function as an alternative approach for transplantation therapy for PD [25]. Organoids, three-dimensional cell culture systems often derived from stem cells, may be a potential source other than grafts to restore DANs and alleviate PD symptoms. By adding supplements required by DANs to support their growth and function, organoids could be a useful source of transplantation. Treatments could also be further optimized by developing patient-derived organoids to personalized medicine.

Moreover, immune modulators such as regulatory T cells could be combined with PINK1 during transplantation to elevate the survival of transplanted dopaminergic neurons. The dysfunction of mitophagy plays a pivotal role in triggering neuroinflammation, which could be especially harmful to dopaminergic neurons in the transplanted graft. Regulatory T cells could thus be used as a suppressor to neuroinflammation by mediating the response to needle trauma, alleviating neuronal and synaptic

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damages [26]. Combining the MTK58 and regulatory T cells in co-transplantation could act as a dual insurance to achieve better clinical outcomes.

### *4.5. Conclusion*

This study has demonstrated a new strategy of enhancing DAN survival in transplantation to the midbrain by modulating PINK1 pathway (Figure 17). We have first verified that non-neuronal defects may be lurking in the microenvironment. We then revealed PINK1 and MTF2 inconsistency during development versus during transplantation. Here we proposed MTK458 as a co-transplanted chemical to activate PINK1 aiming to reduce the effect from non-neuronal signaling, bringing increased efficiency and practicality to PD cell replacement therapy.

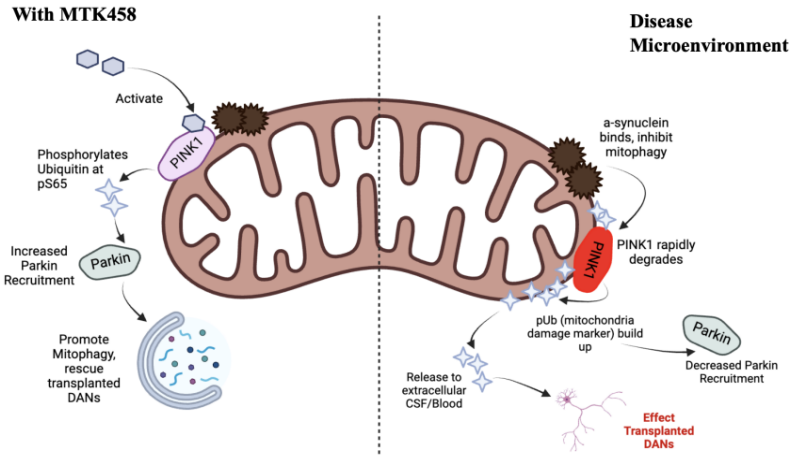


**Figure 17.** Proposed Working Model of MTK58 Enhancing Transplanted DAN Survival Rate by Activating PINK1.

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